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Introduction

The mesolimbic dopamine (DA) system, originating in the ventral tegmental area (VTA), is considered to be a central modulator of self-administration of all drugs of abuse, including ethanol. Specifically, we are interested in understanding the physiology of the GABA_A synapses because the modulation of the GABA_A receptor in the VTA plays a role in many ethanol-related behaviors, including ethanol self-administration. Our hypothesis is that a long-term enhancement of GABAergic synaptic transmission onto DA cells might be a central factor mediating the decrease in DA cell activity during withdrawal from ethanol; to prove it, we plan to characterize the short- and long-term changes of activity at GABA_A receptors onto VTA DA cells after *in vivo* exposure to ethanol. We believe that an important part of reaching the ultimate goal of reducing ethanol consumption will be realized through a better characterization of the physiology of GABA_A receptors in the VTA. A better knowledge of the intrinsic synaptic physiology of VTA GABAergic synapses will enable us to find novel targets aimed at counteracting long-term changes at inhibitory synapses caused by ethanol intake.

Research accomplishments:

This report refers to our first year of funding. We are confident that we have accomplished the goal of specific aim 1. The results of our experiments have been published very recently in a full-length paper in the *Journal of Neuroscience*. I have included a copy of our paper as an appendix, and will refer to it for the figures and for detailed descriptions of our results and their significance.

In specific aim 1, we proposed to characterize whether changes in GABAergic synaptic transmission occurred as a result of exposure to a single *in vivo* injection of ethanol. We proposed to perform these experiments both during acute (1-2 days) and sub-acute (5-30 days) withdrawal from ethanol.

First, we sought to determine whether changes in the probability of GABA release occurred at GABA_A synapses in ethanol-treated mice. In order to accomplish this, we used the paired-pulse stimulation protocol to test for changes in synaptic strength elicited by paired stimuli given at an interval of 50 msec. As explained in our grant proposal, changes in transmitter release would generally affect the paired-pulse ratio (Khazipov et al., 1995; Mennerick and Zorumski, 1995; Debanne et al., 1996; Salin et al., 1996; Stoop and Poo, 1996; Murthy et al., 1997; Gottschalk et al., 1998; Hernandez-Echeagaray et al., 1998; Lessmann and Heumann, 1998; Emmerson and Miller, 1999; Niittykoski et al., 1999; Stanford and Cooper, 1999; Steffensen et al., 1999; Sullivan, 1999; Jiang et al., 2000; Poncer et al., 2000; Cooper and Stanford, 2001; Rozov et al., 2001). However, some inconsistencies have recently been reported for some brain areas (Brody and Yue, 2000; Kraushaar and Jonas, 2000; Waldeck et al., 2000). Figure 1 *a-e* of our paper (see appendix) shows paired-pulse facilitation (PPF) in the saline-treated mice, since the second pulse evoked a GABA_A IPSC (IPSC2) that was significantly larger than the first (IPSC2/IPSC1 = 1.3 ± 0.06 ; $n = 16$). Conversely, ethanol-treated mice exhibited paired

pulse depression (PPD; $\text{IPSC2/IPSC1} = 0.8 \pm 0.02$; $n = 17$; $p < 0.05$). Thus, after a single *in vivo* exposure to ethanol, the paired-pulse protocol resulted in PPD. Then, we decided to determine whether PPF or PPD were dependent on the initial size of the first GABA_A IPSC. Our results show that PPF and PPD were not affected by changes in stimulus intensity (Fig. 1d, appendix). In addition, we analyzed the PPR by dividing the mean of IPSC2 by the mean of IPSC1, and as expected we observed similar results ($p < 0.05$; ethanol, $\text{IPSC2/IPSC1} = 0.7 \pm 0.03$; $n = 17$; saline, $\text{IPSC2/IPSC1} = 1.1 \pm 0.06$; $n = 16$).

Changes in probability of release, function of postsynaptic GABA_A receptors, or a combination of these may be reflected by a variation in PPR toward PPD. We bath applied GABA (100 μM , 3 min) in the presence of the GABA_B receptor antagonist (3-aminopropyl)(diethoxymethyl) phosphinic acid (CGP35348, 100 μM) to determine whether changes in GABA_A receptor function, number, or both occurred following *in vivo* ethanol administration. The amplitude of the inward current elicited by GABA did not differ between ethanol- and saline-treated animals ($n = 4$ for each group; $p > 0.05$; Fig. 1f). These results suggest that modifications in transmitter release, rather than in postsynaptic receptors, occur after an *in vivo* exposure to ethanol. In the next series of experiments, we examined spontaneous GABA_A mIPSCs. Figure 2a-c (see appendix), shows that the frequency of mIPSCs was significantly higher in ethanol- than in saline-treated animals (ethanol, 2.8 ± 0.4 Hz; $n = 9$; saline, 0.7 ± 0.1 Hz; $n = 7$; $p < 0.05$). Furthermore, there was no significant difference in the amplitude of mIPSCs in the two groups (mean amplitudes 30.4 ± 4.2 and 29.2 ± 3.4 pA in saline- and ethanol-treated mice, respectively Fig. 2c,d; appendix, $p > 0.05$). To briefly summarize the experiments described so far, both the paired-pulse protocol and the increased frequency of spontaneous events indicate that the probability of GABA release in the VTA was increased 1 day after a single *in vivo* exposure to ethanol. Previous *in vivo* studies have shown that a decrease in DAergic activity lasts long after somatic signs of withdrawal have disappeared (Diana et al., 1996). Therefore, in order to determine how long the PPD lasted, we collected evoked GABA_A IPSCs by using the paired-pulse protocol both 1 and 2 weeks after the single *in vivo* exposure to ethanol. Figure 1e (see appendix) shows that PPD was still present after 1 week (ethanol, $\text{IPSC2/IPSC1} = 0.9 \pm 0.08$; $n = 9$; $p < 0.05$; saline, $\text{IPSC2/IPSC1} = 1.2 \pm 0.08$; $n = 7$), but that within 2 weeks, the phenomenon had subsided (ethanol, $\text{IPSC2/IPSC1} = 1.1 \pm 0.06$; $n = 8$; $p > 0.05$; saline, $\text{IPSC2/IPSC1} = 1.2 \pm 0.05$; $n = 7$). These data are consistent with the idea that increased probability of GABA release might contribute to the long-lasting hypoactivity of DA neurons that has been observed *in vivo* after ethanol exposure (Diana et al., 1996).

Note: in specific aim 1, we proposed to study changes in GABAergic synaptic transmission both during acute (1-2 days) and sub-acute (5-30 days) withdrawal from ethanol. We did not perform any ratio measurement after day 15 since we did not detect any significant change in the paired-pulse ratio after 2 weeks from the ethanol injection.

In the next series of experiments, we proceeded to investigate in detail whether activation of presynaptic GABA_B receptors was responsible for the observed difference in PPR between the two groups of animals. In the midbrain, GABA_B receptors are present presynaptically and postsynaptically, and it has been shown that the activation of presynaptic GABA_B receptors causes inhibition of GABA_A IPSCs (Johnson and North, 1992b; Hausser and Yung, 1994). Therefore, we measured the PPR in the presence of the

GABA_B receptor antagonist CGP35348 (100 μ M, 5 min). Figure 4, *a* and *b* (see appendix), shows that CGP35348 significantly shifted the PPD to PPF in ethanol-treated animals (IPSC2/IPSC1 = 0.7 ± 0.04 - 1.2 ± 0.08 ; $n = 10$; $p < 0.05$) by increasing the amplitude of the second evoked GABA_A IPSC (IPSC2, $140 \pm 9\%$; data not shown) without affecting either GABA_A IPSC in the saline-treated animals (IPSC2/IPSC1 = 1.5 ± 0.1 - 1.4 ± 0.2 ; $n = 5$; $p > 0.05$).

In both groups of mice, the frequency and the amplitude of mIPSCs were unaffected by CGP35348 (100 μ M, 5 min) (frequency: ethanol, 2.9 ± 0.2 - 2.6 ± 0.3 Hz; $n = 7$; saline, 1.1 ± 0.1 - 1.1 ± 0.1 Hz; $n = 7$; amplitude: ethanol, 33.6 ± 1.8 - 33 ± 2.9 pA; $n = 5$; saline, 33.2 ± 2.1 - 26.9 ± 2.6 pA; $n = 7$; Fig. 4*c,d*). In conclusion, the PPD observed in the ethanol-treated mice could result from an increased probability of GABA release in the ethanol-treated mice, and this might in turn lead to activation of presynaptic GABA_B receptors. It is also possible that the sensitivity of presynaptic GABA_B receptors might be enhanced in the ethanol-treated animals. In order to find out, we compared the inhibition caused by the GABA_B receptor agonist baclofen in slices from both saline- and ethanol-treated animals by testing the differences in sensitivity of presynaptic GABA_B receptors. The results shown in figure 5 *a* and *b* (see appendix) show that the concentration-response curves to baclofen were similar in the saline- and ethanol-treated mice (baclofen 0.1 μ M: ethanol, $22.3 \pm 8.9\%$; $n = 5$; saline, $25.4 \pm 3.1\%$; $n = 5$; baclofen 1 μ M: ethanol, $42.8 \pm 12.3\%$; $n = 5$; saline, $44.5 \pm 2.3\%$; $n = 5$; baclofen 10 μ M: ethanol, $70.6 \pm 11.8\%$; $n = 5$; saline, $72.7 \pm 7.2\%$; $n = 5$). Thus, the increase in the IPSC2 observed in the presence of CGP35348 in ethanol-treated animals did not result from altered sensitivity of GABA_B receptors to endogenous GABA. Finally, when we applied a high dose of baclofen (10 μ M; Fig. 5*c,d*, see appendix), such a dose reverted the PPD to PPF in ethanol-treated mice (IPSC2/IPSC1 = 0.7 ± 0.02 - 1.2 ± 0.1 ; $n = 5$; $p < 0.05$), but it produced an insignificant increase in PPF in saline-treated animals (IPSC2/IPSC1 = 1.2 ± 0.1 - 1.3 ± 0.1 ; $n = 5$). These results further support the hypothesis that GABA levels are increased after ethanol exposure, leading to spillover onto presynaptic GABA_B receptors, whose activation leads to inhibition of GABA release.

Note: Because changes in the paired-pulse ratio and changes in the probability of release are generally linked, we expected baclofen to increase PPF in the saline group. However, we detected a non-significant increase in the PPF during the application of baclofen at this concentration (IPSC2/IPSC1 = 1.2 ± 0.09 - 1.3 ± 0.02 ; $n = 5$; $p > 0.05$) and at 1 μ M (IPSC2/IPSC1 = 1.1 ± 0.07 - 1.2 ± 0.08 ; $n = 5$; $p > 0.05$) in the saline-treated animals. This indicates that the PPR measure is not sensitive enough in this range to detect a decrease in GABA release, possibly as a result of a ceiling effect.

In their 1997 work, Bonci and Williams described an increase in probability of GABA release during acute withdrawal from chronic morphine in several brain regions, including the VTA, as well as its dependence upon cAMP in the VTA. We decided to examine the possibility that the cAMP-dependent pathway was modified in the ethanol-treated animals since ethanol and other drugs of abuse are known to modulate the camp-protein kinase A (PKA) cascade within the mesolimbic system (Hoffman and Tabakoff, 1990; Self et al., 1998; Spanagel and Weiss, 1999). To do so, we directly activated adenylyl cyclase (AC) by bath applying forskolin. Forskolin (10 μ M, 10 min) augmented

the IPSC1 in saline-treated animals (Fig. 6a, *see appendix*, $111.6 \pm 17.4\%$; $n = 5$; $p < 0.05$) but had no effect on the amplitude of the IPSC1 in ethanol-treated mice ($23.1 \pm 13.3\%$; $n = 5$). Thus, application of forskolin decreased the paired-pulse ratio toward depression in slices from saline-treated animals (Fig. 6b; $\text{IPSC2/IPSC1} = 1.4 \pm 0.1$ - 0.9 ± 0.1 ; $n = 5$; $p < 0.05$), but was without effect in slices from ethanol-treated animals ($\text{IPSC2/IPSC1} = 0.8 \pm 0.05$ - 0.7 ± 0.1 ; $n = 5$). This supports the idea that activation of AC increased the probability of GABA release. In addition, the frequency of spontaneous mIPSCs was also significantly increased by forskolin in saline-treated mice (0.7 ± 0.1 - 1.7 ± 0.3 Hz; $n = 7$; $p < 0.05$; Fig. 6c), but not in the ethanol-treated animals (2.6 ± 0.7 - 2.9 ± 1.0 Hz; $n = 8$; Fig. 6c). There was no significant difference in the amplitude of the mIPSCs in the absence or presence of forskolin in slices from either group of animals (saline, 29.4 ± 2.5 - 31 ± 2.9 pA; $n = 7$; ethanol, 34 ± 4.2 - 26.5 ± 1.7 pA; $n = 8$; Fig. 6d). To rule out the possibility of nonspecific effects of forskolin, we tested 1,9-dideoxyforskolin, an inactive analog of forskolin (Seamon and Daly, 1985). Superfusion of 1,9-dideoxyforskolin (10 μ M, 10 min) had no effect on IPSCs in either group of animals (7.7 ± 3.3 and $1.1 \pm 2.6\%$ in ethanol- and saline-treated mice, respectively; $n = 4$ for each group; data not shown). Taken together, these results suggest that a saturation of AC might occur after a single *in vivo* exposure to ethanol. Because PKA activity is altered by intracellular cAMP levels, in the next series of experiments we tested the effect of *N*-[2(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H89), which inhibits PKA in a competitive manner against ATP (Chijiwa et al., 1990). Superfusion of H89 (10 μ M, 20 min) significantly reduced the amplitude of IPSC1 in ethanol-treated mice ($38.6 \pm 5.4\%$; $n = 5$; $p < 0.05$; Fig. 7a, *see appendix*), but had no effect in slices from control animals ($3.4 \pm 8.1\%$; $n = 5$). The subsequent application of forskolin (10 μ M, 10 min) in the presence of H89, used to test the activity of H89 in control animals, did not change the amplitude of the IPSC1 in either group (Fig. 7a, *see appendix*). Furthermore, H89, by reducing the size of IPSC1, shifted the PPD to PPF in slices from ethanol-treated animals ($\text{IPSC2/IPSC1} = 0.8 \pm 0.02$ - 1.1 ± 0.01 ; $n = 5$; $p < 0.05$; Fig. 7b, *see appendix*). Consistent with these results, H89 reduced the frequency of spontaneous mIPSC in the ethanol-treated animals (2.6 ± 0.1 - 1.6 ± 0.1 Hz; $n = 6$; $p < 0.05$; Fig. 7c, *see appendix*), but not in the saline-treated animals (0.9 ± 0.1 - 0.8 ± 0.1 Hz; $n = 5$); in the presence of H89, the amplitude of spontaneous mIPSCs was not changed in either saline-treated mice (31.7 ± 5.2 - 31.3 ± 2.6 pA; $n = 5$) or ethanol-treated mice (28.7 ± 3.1 - 24.8 ± 2.9 pA; $n = 6$). These experiments indicate that a single exposure to ethanol enhances PKA activity, and that such a phenomenon increases the probability of GABA release in the VTA. In our final series of experiments, we decided to test whether changes in ethanol self-administration were produced by the same single *in vivo* ethanol exposure used for our electrophysiological experiments. Behavioral studies reported that a single *in vivo* exposure to ethanol produced an increase in subsequent self-administration of ethanol in rodents (Camarini et al., 2000; Files et al., 2000). Additionally, because GABA_A agonists facilitate acquisition of voluntary ethanol drinking in rats (Smith et al., 1992; Nowak et al., 1998), activation of GABA_A receptors plays a role in ethanol self-administration. Therefore, mice that underwent the same experimental protocol were studied for ethanol consumption. To perform these experiments, 24 hr after a single exposure to ethanol (2 gm/kg, i.p.), mice were given a two-bottle choice test with two concentrations of ethanol versus water for 5 d at each

concentration (2 and 5% v/v, respectively). In order to allow the animals to acclimate to the taste of ethanol, we began with the lower concentration for 5 d. As reported previously (Camarini et al., 2000), C57BL/6J mice pretreated with ethanol consumed more ethanol than saline controls after 10 d of housing in the continuous access situation (Fig. 3a, *see appendix*). We found that 10 d after the acute exposure, the mean ethanol intake was 9.3 ± 0.6 and 7.4 ± 0.4 gm/kg for ethanol-treated ($n = 8$) and saline-treated ($n = 8$) mice, respectively ($p < 0.05$). Although both groups preferred water to ethanol, ethanol-treated mice showed an increased preference for ethanol ($46.9 \pm 0.5\%$; $n = 8$; $p < 0.05$; Fig. 3b, *see appendix*) when compared with saline controls ($41.1 \pm 1.5\%$; $n = 8$). However, because increased ethanol intake observed in ethanol-treated mice may be affected by differential absorption, distribution, or clearance of ethanol, we measured blood ethanol concentrations 10-90 min after administration of ethanol (4 gm/kg, i.p.). Figure 3c of the appendix shows that ethanol clearance did not differ between the two groups of animals, although it showed lower blood ethanol levels than commonly reported (Hodge et al., 1999; Thiele et al., 2000; Wand et al., 2001). One possible explanation for such low levels might be the faster metabolism of younger mice used for the present study compared with the 2- to 4-month-old mice tested in other studies (Hodge et al., 1999; Thiele et al., 2000; Wand et al., 2001). In conclusion, taken together, our results support the hypothesis that an increased GABAergic transmission in the VTA may be involved in facilitating or maintaining ethanol consumption.

Key research accomplishment:

- 1) Our data demonstrate that a single *in vivo* injection of ethanol produces a long-term potentiation of GABAergic synapses onto dopaminergic neurons in the VTA.
- 2) Such a potentiation lasts for a week after the ethanol injection, and is caused by a cAMP-PKA dependent mechanism.
- 3) We have also provided evidence that a single *in vivo* injection of ethanol also increases subsequent ethanol consumption.

Conclusions:

To our knowledge, this is the first study that directly correlates a synaptic modification at GABA_A receptors with an abnormal ethanol-dependent behavior, namely ethanol self-administration.

Our results suggest that increased GABA_A receptor activation in the VTA plays a central role in increasing ethanol self-administration, and indicate that this cAMP-PKA-dependent

plasticity occurring at GABA_A synapses might represent an important crucial signaling event underlying increased ethanol consumption.

Reportable outcomes:

One Full-length article:

- 1) "Long-lasting potentiation of GABAergic synapses in dopamine neurons after a single in vivo ethanol exposure". *J.Neuroscience*, 2002, 22 (6) 2074-82.

Abstracts:

- 1) Effects of ethanol withdrawal on gabaergic synapses in dopamine neurons. M. Melis, M.A. Ungless, & A. Bonci. Society for Neuroscience, Annual Conference, San Diego, 2001.
- 2) Effects of a single exposure to ethanol in mice: a behavioral and electrophysiological study. R. Camarini, M. Melis, C.W. Hodge & A. Bonci. Society for Neuroscience, Annual Conference, San Diego, 2001.
- 3) M. Melis, M.A. Ungless, A. Bonci (2001) The effects of alcohol withdrawal on synaptic transmission of VTA DA neurons in C57Bl/6J mice. 24th Annual Meeting of Research Society on Alcoholism, Montreal, June 23-28 2001.

Presentations:

- Invited speaker in the seminar series at UT Southwestern, Dallas, TX (February 2002)
- Invited speaker at the Annual Meeting of Research Society for Alcoholism (June 2002)
- Invited speaker in the seminar series at UCSD, The Scripps Institute, San Diego (October 2002)
- Invited speaker at Society for Neuroscience Symposium (November 2002)
- Invited speaker at American College of Neuropsychopharmacology Symposium (December 2002).

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Long-Lasting Potentiation of GABAergic Synapses in Dopamine Neurons after a Single *In Vivo* Ethanol Exposure

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The mesolimbic dopamine (DA) system originating in the ventral tegmental area (VTA) is involved in many drug-related behaviors, including ethanol self-administration. In particular, VTA activity regulating ethanol consummatory behavior appears to be modulated through GABA_A receptors. Previous exposure to ethanol enhances ethanol self-administration, but the mechanisms underlying this phenomenon are not well understood. In this study, we examined changes occurring at GABA synapses onto VTA DA neurons after a single *in vivo* exposure to ethanol. We observed that evoked GABA_A IPSCs in DA neurons of ethanol-treated animals exhibited paired-pulse depression (PPD) compared with saline-treated animals, which exhibited paired-pulse facilitation (PPF). Furthermore, PPD was still present 1 week after the single exposure to ethanol. An increase in frequency of spontaneous miniature GABA_A IPSCs (mIPSCs) was also observed in the ethanol-treated animals. Additionally, the GABA_B receptor antagonist (3-aminopropyl)

(diethoxymethyl) phosphinic acid shifted PPD to PPF, indicating that presynaptic GABA_B receptor activation, likely attributable to GABA spillover, might play a role in mediating PPD in the ethanol-treated mice. The activation of adenylyl cyclase by forskolin increased the amplitude of GABA_A IPSCs and the frequency of mIPSCs in the saline- but not in the ethanol-treated animals. Conversely, the protein kinase A (PKA) inhibitor *N*-[α -(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide significantly decreased both the frequency of spontaneous mIPSCs and the amplitude of GABA_A IPSCs in the ethanol-treated mice but not in the saline controls. The present results indicate that potentiation of GABAergic synapses, via a PKA-dependent mechanism, occurs in the VTA after a single *in vivo* exposure to ethanol, and such potentiation might be a key synaptic modification underlying increased ethanol intake.

Key words: ventral tegmental area; ethanol; probability of GABA release; presynaptic plasticity; cAMP; PKA

The mesolimbic dopamine (DA) system originates in the ventral tegmental area (VTA), and it projects to structures associated with the limbic system, primarily the nucleus accumbens (NAcc), the amygdala, the hippocampus, and the prefrontal cortex (Fuxe et al., 1974; Oades and Halliday, 1987). A growing body of evidence implicates the mesolimbic DA system in the regulation of ethanol self-administration (Rassnick et al., 1993a; Samson et al., 1993; Ng and George, 1994; Koob et al., 1998; McBride et al., 1999; Kaczmarek and Kiefer, 2000; Nowak et al., 2000). An important role of DA in ethanol reinforcement has been suggested by studies showing that DA receptor antagonists, injected systemically or directly into the terminal regions of the mesolimbic DA system, decrease lever pressing for ethanol (Samson et al., 1993; Ng and George, 1994). Furthermore, a variety of pharmacological manipulations within this pathway, affecting the activity of DA neurons, produced changes in ethanol consumption, suggesting that DA neuronal activity within the VTA may be important for maintaining ethanol consummatory behavior (Rassnick et

al., 1993a; Ng and George, 1994; Kaczmarek and Kiefer, 2000; Nowak et al., 2000). In fact, a marked reduction of the spontaneous activity of mesolimbic DA neurons (Diana et al., 1993; Bailey et al., 1998), resulting in decreased extracellular DA levels in NAcc (Diana et al., 1993), has been observed during acute withdrawal from chronic ethanol. Moreover, the fact that ethanol intake in dependent rats greatly exceeds that of nondependent rats during acute withdrawal, and that increased self-administration restores DA levels to normal in NAcc, suggests that decreased DA levels may trigger ethanol-seeking behavior (Weiss et al., 1996). The above-mentioned studies indicate that changes in activity of VTA DA cells, correlated with extracellular DA levels in the NAcc, might regulate ethanol consumption (McBride et al., 1995; Weiss et al., 1996; Hodge et al., 1997; Ikemoto et al., 1997), and accordingly, DA antagonists impair alcohol self-administration. In the midbrain dopamine systems, GABAergic neurons exert an inhibitory control on DA neurons (Johnson and North, 1992b; Hauser and Yung, 1994; Paladini et al., 1999). Therefore, hyperactivity of VTA GABA cells observed during acute withdrawal from chronic ethanol (Gallegos et al., 1999) could account, at least in part, for the reduced DAergic activity. Because the hypofunction of the DAergic system outlasts the somatic signs of acute withdrawal (Diana et al., 1996), such an increase of GABAergic synaptic transmission might not only represent a functional correlate of acute withdrawal from ethanol but also play a role in both short- and long-term consequences produced by ethanol exposure.

Interestingly, the influence of the initial exposure to ethanol and the patterns of its subsequent consumption have been ob-

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served in both humans and laboratory animals (Haertzen et al., 1983; Camarini et al., 2000; Files et al., 2000). Unfortunately, the relationship between the reinforcing quality of the first experience and subsequent habits of ethanol consumption is still unclear. Whether this change in behavior is attributable to a reduced sensitivity to the stimulant effects of ethanol (Phillips et al., 1995) or a blockade of the development of ethanol-induced conditioned taste aversion (Risner and Cunningham, 1995) remains to be elucidated. Because both systemic and intra-VTA administration of GABA_A receptor agonists facilitate, whereas antagonists decrease, the acquisition of voluntary ethanol drinking in rats (Smith et al., 1992; Nowak et al., 1998), the GABAergic transmission within the VTA might play an important role.

Although the acute effects of ethanol in the mesolimbic system have been studied extensively (Brodie et al., 1990, 1999; Nie et al., 1993, 1994; Brodie and Appel, 1998, 2000; Steffensen et al., 2000), there are no studies directly examining whether synaptic changes occur in the VTA after exposure to ethanol.

To address this issue, we studied GABA_A-mediated IPSCs in VTA DA neurons 24 hr after a single injection of either ethanol (2 gm/kg, i.p.) or saline.

MATERIALS AND METHODS

Slice preparation. The preparation of VTA slices was as described previously (Thomas et al., 2000). Briefly, C57BL/6J mice (21–35 d; Charles River, Hollister, CA) were anesthetized with halothane and killed. A block of tissue containing the midbrain was sliced in the horizontal plane (230 μ m) with a vibratome (Leica, Nussloch, Germany) in ice-cold low-Ca²⁺ solution containing (in mM): 126 NaCl, 1.6 KCl, 1.2 NaH₂PO₄, 1.2 MgCl₂, 0.625 CaCl₂, 18 NaHCO₃, and 11 glucose. Slices (two per animal) were transferred in a holding chamber with a bicarbonate-buffered solution (32–34°C) saturated with 95%O₂ and 5%CO₂ containing (in mM): 126 NaCl, 1.6 KCl, 1.2 NaH₂PO₄, 1.2 MgCl₂, 2.4 CaCl₂, 18 NaHCO₃, and 11 glucose. Slices were allowed to recover for at least 1 hr before being placed in the recording chamber and superfused with the bicarbonate-buffered solution (32–34°C) saturated with 95%O₂ and 5%CO₂.

Whole-cell recording. Only one cell for each experimental procedure was recorded per mouse. Cells were visualized with an upright microscope with infrared illumination, and whole-cell voltage-clamp recordings were made by using an Axopatch 1D amplifier (Axon Instruments, Foster City, CA). All GABA_A IPSC recordings were made with electrodes filled with an internal solution containing the following (in mM): 144 KCl, 1 CaCl₂, 3.45 K₂BAPTA, 10 HEPES, 2 Mg₂ATP, and 0.25 Mg₂GTP, pH 7.2–7.4. Experiments were begun only after series resistance had stabilized (typically 15–40 M Ω). Series and input resistance were monitored continuously on-line with a 4 mV depolarizing step (25 msec). Data were filtered at 2 kHz, digitized at 10 kHz, and collected on-line with acquisition software (Igor Pro, Lake Oswego, OR). Because of the composition of the internal solution, the GABA_A IPSCs were inward at a membrane potential of -70 mV and were completely blocked by picrotoxin (100 μ M). DA cells were identified by the presence of a large I_h current (Johnson and North, 1992a) that was assayed immediately after break-in, using a series of incremental 10 mV hyperpolarizing steps from a holding potential of -70 mV. A bipolar stainless steel stimulating electrode was placed 100 μ m rostral to the recording electrode and was used to stimulate at a frequency of 0.1 Hz. Neurons were voltage-clamped at a membrane potential of -70 mV. All GABA_A IPSCs were recorded in the presence of 2-amino-5-phosphonopentanoic acid (AP5; 100 μ M), 6-cyano-2,3-dihydroxy-7-nitro-quinoxaline (10 μ M), strychnine (1 μ M), and eticlopride (100 nM) to block NMDA, AMPA, glycine, and dopamine D2-mediated synaptic currents, respectively. As described previously (Bonci and Williams, 1997), this solution had no effect on the holding current of the dopamine cells. The amplitudes of IPSCs were calculated by taking a 1 msec window around the peak of the IPSC and comparing this with the 5 msec window immediately before the stimulus artifact. Paired stimuli were given with an interstimulus interval of 50 msec, and the ratio between the second and the first IPSCs was calculated and averaged for a 10 min baseline. Drugs were applied in known concentrations to the superfusion medium. The spontaneous

miniature IPSCs (mIPSC) were collected in the presence of lidocaine (500 μ M) and analyzed (120 sweeps for each condition, 1 sec/sweep) using Mini Analysis program (Synaptosoft). To accurately determine the mIPSC amplitude, only mIPSCs that were >8 pA were accepted for analysis. The choice of this cutoff amplitude for acceptance of mIPSCs was made to obtain a high signal-to-noise ratio.

Alcohol self-administration. Sixteen C57BL/6J mice (Charles River Laboratories, Wilmington, MA) were housed individually in polycarbonate cages, with food and water available *ad libitum*, and habituated to their home cage for 1 week before the experiment. The colony room was maintained on a 12 hr light/dark cycle with lights on at 6 A.M. All experimental procedures were conducted under institutional and National Institutes of Health guidelines. Oral ethanol self-administration was examined using a two-bottle choice protocol (Phillips et al., 1998; Hodge et al., 1999). Mice were offered the choice between 2% (v/v) ethanol versus water for 5 d. The ethanol concentration was increased to 5%, and the ethanol consumption was measured for 5 more days. Fluid volumes consumed were recorded every day, and the bottle positions were alternated daily. Each day, the mice were weighed, and then the ethanol consumption was calculated as grams of ethanol per kilogram.

Blood alcohol determination. Blood tail collection of 3- to 4-week-old mice did not provide enough volume for measurement; therefore, blood ethanol concentration was measured by drawing a 40 μ l blood sample from the trunk. Twenty-four hours after either ethanol (2 gm/kg, i.p.) or saline exposure, blood samples were collected at 10, 30, 60, and 90 min after an intraperitoneal 4 gm/kg injection of ethanol (four or five mice were used for each group and for every time point). Blood plasma was extracted with trichloroacetic acid, and plasma ethanol content was measured using a 332 alcohol diagnostic kit (Sigma, St. Louis, MO).

Results in the text and figures are presented as the mean \pm SEM. Results between groups were compared using a *t* test, either paired or unpaired where appropriate; *p* < 0.05 was taken as indicating statistical significance.

RESULTS

In the present study, we investigated the properties of GABA_A IPSCs recorded in VTA DA cells from mice that received a single *in vivo* injection of ethanol (2 gm/kg, i.p.) or saline the day before the recordings.

First, to determine whether changes in the probability of GABA release occurred at these synapses in ethanol-treated mice, we use the paired-pulse stimulation protocol to test for changes in synaptic strength elicited by paired stimuli given at an interval of 50 msec. It has been shown that changes in transmitter release would generally affect the paired pulse ratio (Khazipov et al., 1995; Mennerick and Zorumski, 1995; Debanne et al., 1996; Salin et al., 1996; Stoop and Poo, 1996; Murthy et al., 1997; Gottschalk et al., 1998; Hernandez-Echeagaray et al., 1998; Lessmann and Heumann, 1998; Emmerson and Miller, 1999; Niittykoski et al., 1999; Stanford and Cooper, 1999; Steffensen et al., 1999; Sullivan, 1999; Jiang et al., 2000; Poncer et al., 2000; Yun et al., 2000; Cooper and Stanford, 2001; Rozov et al., 2001). Although some inconsistencies have recently been reported for some brain areas (Brody and Yue, 2000; Kraushaar and Jonas, 2000; Waldeck et al., 2000), it is well established that changes in transmitter release affect the paired-pulse ratio (PPR) in the VTA (Bonci and Williams, 1997; Manzoni and Williams, 1999). In slices from saline-treated mice, we observed paired-pulse facilitation (PPF), with the second pulse evoking a GABA_A IPSC (IPSC2) that was significantly larger than the first (Fig. 1*a,e*; IPSC2/IPSC1 = 1.3 ± 0.06 ; *n* = 16). Conversely, ethanol-treated mice exhibited paired pulse depression (PPD; Fig. 1*b,e*; IPSC2/IPSC1 = 0.8 ± 0.02 ; *n* = 17; *p* < 0.05). Thus, after a single *in vivo* exposure to ethanol, the paired-pulse protocol resulted in PPD (Fig. 1*c–e*). PPF or PPD did not depend on the size of the first GABA_A IPSC (IPSC1; Fig. 1*c*) and was not affected by changes in stimulus intensity (Fig. 1*d*). In addition, we analyzed the PPR by dividing the mean of IPSC2 by the mean of IPSC1, and we find

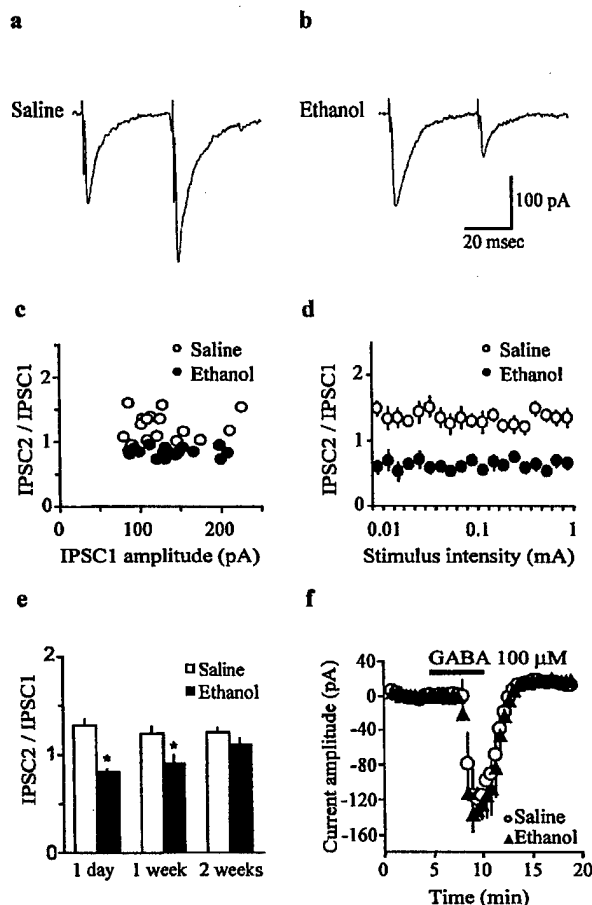


Figure 1. Increased probability of GABA release 24 hr after a single *in vivo* exposure to ethanol. GABA_A IPSCs from ethanol-treated mice show PPD compared with saline controls, which show PPF. *a*, *b*, Examples of recordings from saline-treated (*a*) and ethanol-treated (*b*) animals. *c*, No correlation was found between the amplitude of IPSC1 and the IPSC2/IPSC1 ratio in both saline-treated mice ($n = 16$) and ethanol-treated mice ($n = 17$). *d*, The IPSC2/IPSC1 ratio is independent of the stimulus strength. Results are the average from four cells in each group of animals. *e*, PPD in ethanol-treated mice is a long-lasting phenomenon. The bar graph shows the average IPSC2/IPSC1 ratio (mean \pm SEM) of saline- and ethanol-treated mice after 1 d ($n = 16$ and 17 for saline and ethanol, respectively; $*p < 0.05$), 1 week ($n = 9$ per group; $*p < 0.05$), and 2 weeks ($n = 8$ per group; $p > 0.05$) after ethanol pre-exposure. *f*, Ethanol pre-exposure does not change either number or function of postsynaptic GABA_A receptors. Bath application of GABA (100 μ M, 3 min) in the presence of GABA_B receptor antagonist CGP 35348 (100 μ M) elicited a similar current (30 sec bins) in both groups of animals ($n = 4$; $p > 0.05$) when neurons were voltage-clamped at -70 mV.

this method yields similar results ($p < 0.05$; ethanol, IPSC2/IPSC1 = 0.7 ± 0.03 ; $n = 17$; saline, IPSC2/IPSC1 = 1.1 ± 0.06 ; $n = 16$).

A change in PPR toward PPD might reflect changes in probability of release, function of postsynaptic GABA_A receptors, or a combination of these. To determine whether changes in GABA_A receptor function, number, or both occurred after *in vivo* ethanol administration, we bath applied GABA (100 μ M, 3 min) in presence of the GABA_B receptor antagonist (3-aminopropyl) diethoxymethyl phosphinic acid (CGP35348) (100 μ M). The amplitude of the inward current elicited by GABA did not differ between ethanol- and saline-treated animals ($n = 4$ for each group; $p > 0.05$; Fig. 1*f*). These results suggest that modifications

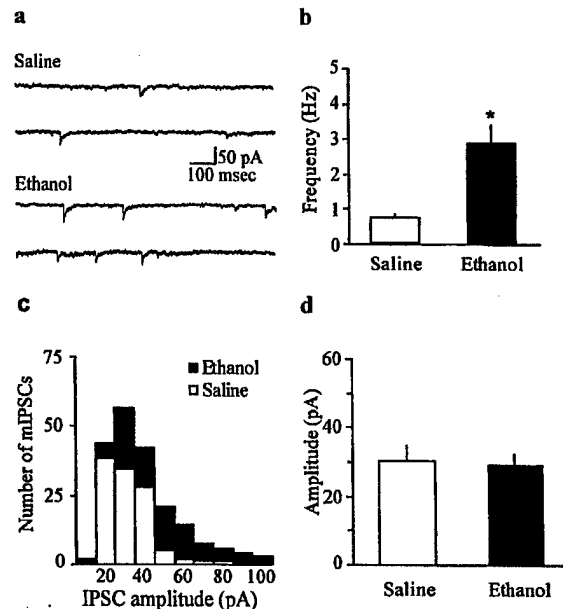


Figure 2. Ethanol pre-exposure increased the frequency, but not amplitude, of spontaneous mIPSCs. *a*, Samples of mIPSCs from saline-treated mice (top traces) and ethanol-treated mice (bottom traces). *b*, Bar graph showing the average (mean \pm SEM) frequency for saline-treated animals ($n = 7$) and ethanol-treated animals ($n = 9$; $*p < 0.05$). *c*, Bar graph (10 pA bins) showing an amplitude histogram of mIPSCs for ethanol-treated ($n = 9$) versus saline-treated ($n = 7$) mice. *d*, Bar graph showing the average (mean \pm SEM) amplitude for saline-treated animals ($n = 7$) and ethanol-treated animals ($n = 9$).

in transmitter release, rather than in postsynaptic receptors, occur after an *in vivo* exposure to ethanol.

To further test this possibility, we examined spontaneous GABA_A mIPSCs. Figure 2, *a–c*, shows that the frequency of mIPSCs was significantly higher in ethanol- than in saline-treated animals (ethanol, 2.8 ± 0.4 Hz; $n = 9$; saline, 0.7 ± 0.1 Hz; $n = 7$; $p < 0.05$). Furthermore, there was no significant difference in the amplitude of mIPSCs in the two groups, with mean amplitudes of 30.4 ± 4.2 and 29.2 ± 3.4 pA in saline- and ethanol-treated mice, respectively (Fig. 2*d*; $p > 0.05$). Because an increase in frequency but not amplitude of mIPSCs is generally thought to reflect a presynaptic increase in probability of transmitter release (Malenka and Nicoll, 1999), both the paired pulse protocol and the increased frequency of spontaneous events indicate that the probability of GABA release in the VTA was increased 1 d after a single exposure to ethanol.

Previous *in vivo* studies have shown that reduced DAergic activity persists long after somatic signs of withdrawal have subsided (Diana et al., 1996). Therefore, we collected evoked GABA_A IPSCs by using the paired-pulse protocol both 1 and 2 weeks after the single *in vivo* exposure to ethanol to determine how long the PPD lasted. Figure 1*e* shows that PPD was still present after 1 week (ethanol, IPSC2/IPSC1 = 0.9 ± 0.08 ; $n = 9$; $p < 0.05$; saline, IPSC2/IPSC1 = 1.2 ± 0.08 ; $n = 7$), but that within 2 weeks, the phenomenon had subsided (ethanol, IPSC2/IPSC1 = 1.1 ± 0.06 ; $n = 8$; $p > 0.05$; saline, IPSC2/IPSC1 = 1.2 ± 0.05 ; $n = 7$). These data are consistent with the idea that increased probability of GABA release might contribute to the long-lasting hypoactivity of DA neurons that has been observed *in vivo* after ethanol exposure (Diana et al., 1996).

Behavioral studies reported that a single *in vivo* exposure to

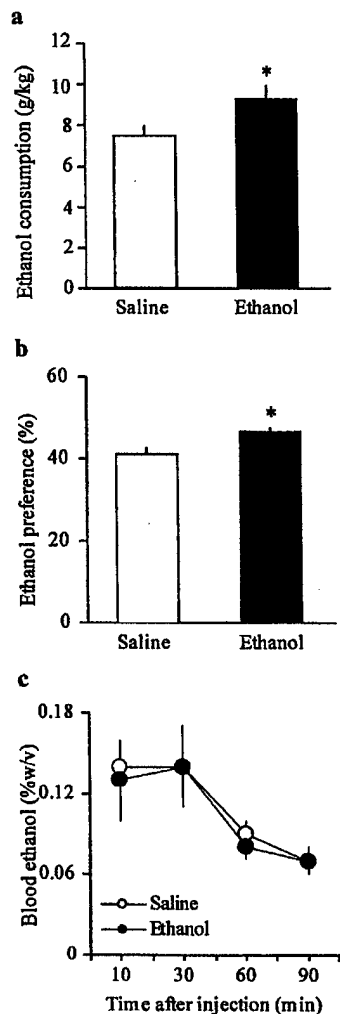


Figure 3. One week after the pre-exposure, ethanol-treated mice show increased ethanol intake and preference compared with saline controls. *a*, Voluntary 24 hr ethanol consumption (grams per kilogram) in C57BL/6J mice pretreated with ethanol ($n = 8$; $*p < 0.05$) and saline ($n = 8$). *b*, Ethanol preference, calculated as $100 \times$ milliliters of ethanol per total milliliters consumed. C57BL/6J mice pretreated with ethanol demonstrated a significant increase in ethanol preference ($n = 8$; $*p < 0.05$) when compared with saline controls ($n = 8$). *c*, Blood ethanol clearance after acute administration of ethanol (4 gm/kg, i.p.) did not differ between ethanol- and saline-treated mice. Data (mean \pm SEM) represent four animals per each group at every time point.

ethanol produced an increase in subsequent self-administration of ethanol in rodents (Camarini et al., 2000; Files et al., 2000). In addition, activation of GABA_A receptors plays a role in ethanol self-administration, because GABA_A agonists facilitate acquisition of voluntary ethanol drinking in rats (Smith et al., 1992; Nowak et al., 1998). We therefore decided to measure the ethanol consumption in mice that underwent the same experimental protocol. To perform these experiments, 24 hr after a single exposure to ethanol (2 gm/kg, i.p.), mice were given a two-bottle choice test with two concentrations of ethanol versus water for 5 d at each concentration (2 and 5% v/v, respectively). We began with the lower concentration for 5 d to allow the animals to acclimate to the taste of ethanol. As reported previously (Camarini et al., 2000), C57BL/6J mice pretreated with ethanol consumed more ethanol than saline controls after 10 d of housing in the contin-

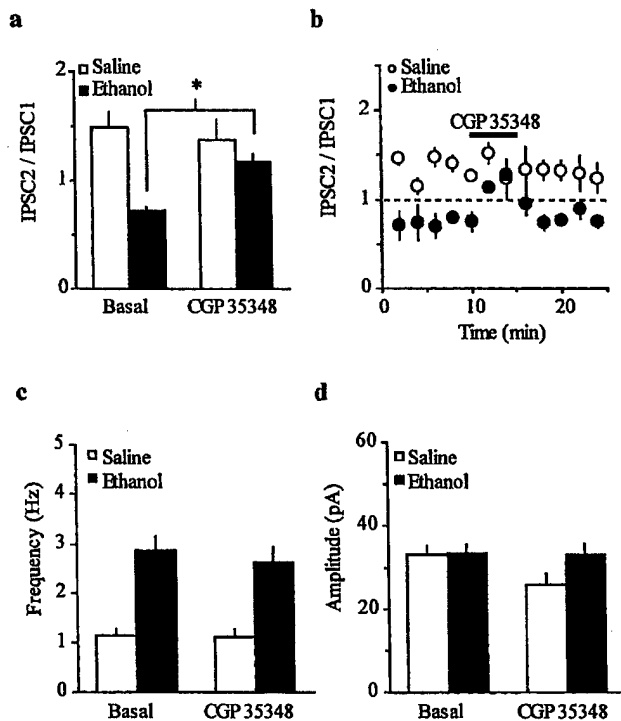


Figure 4. Effect of the GABA_B receptor antagonist CGP35348 (100 μ M) on IPSC2/IPSC1 ratio and spontaneous mIPSCs. *a*, CGP35348 (100 μ M, 5 min) shifts the PPD to PPF in ethanol-treated mice ($n = 10$; $*p < 0.05$), without affecting the PPF in saline-treated mice ($n = 5$). *b*, The IPSC2/IPSC1 ratio is plotted as a function of time in cells recorded from saline- and ethanol-treated mice and normalized against the mean of the first 10 min for each cell. *c*, CGP35348 does not change the frequency of mIPSCs in either group ($n = 7$ per each group). *d*, No changes in amplitude of spontaneous mIPSCs were found in either group ($n = 7$ per each group).

uous access situation (Fig. 3*a*). We found that 10 d after the acute exposure, the mean ethanol intake was 9.3 ± 0.6 and 7.4 ± 0.4 gm/kg for ethanol-treated ($n = 8$) and saline-treated ($n = 8$) mice, respectively ($p < 0.05$). Although both groups preferred water to ethanol, ethanol-treated mice showed an increased preference for ethanol ($46.9 \pm 0.5\%$; $n = 8$; $p < 0.05$; Fig. 3*b*) when compared with saline controls ($41.1 \pm 1.5\%$; $n = 8$). However, because differential absorption, distribution, or clearance of ethanol may contribute to the increased ethanol intake observed in ethanol-treated mice, we measured blood ethanol concentrations 10–90 min after administration of ethanol (4 gm/kg, i.p.). Figure 3*c* shows that ethanol clearance did not differ between the two groups of animals, although it showed lower blood ethanol levels than commonly reported (Hodge et al., 1999; Thiele et al., 2000; Wand et al., 2001). One possible explanation for such low levels might be the faster metabolism of younger mice used for the present study compared with the 2- to 4-month-old mice tested in other studies (Hodge et al., 1999; Thiele et al., 2000; Wand et al., 2001). Nevertheless, taken together, these results support the hypothesis that an increased GABAergic transmission in the VTA may be involved in facilitating or maintaining ethanol consumption (Smith et al., 1992; Nowak et al., 1998).

The observed difference in PPR between the two groups of animals might result indirectly from activation of presynaptic receptors. In the midbrain, GABA_B receptors are present presynaptically and postsynaptically, and it has been shown that the activation of presynaptic GABA_B receptors causes inhibition of

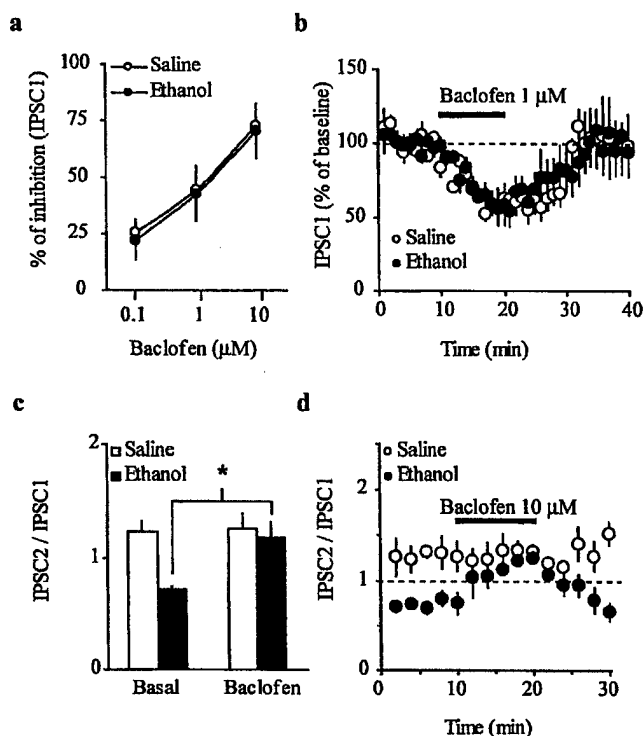


Figure 5. Effect of the GABA_B receptor agonist baclofen on evoked IPSCs. *a*, Concentration–response curve for baclofen measuring the amplitude of IPSC1 from saline- and ethanol-treated animals ($n = 5$ per each group at all doses tested). *b*, Baclofen ($1 \mu\text{M}$, 10 min) decreases the amplitude of IPSC1 in both saline- and ethanol-treated mice to the same extent ($n = 5$ per each group). *c*, Baclofen ($10 \mu\text{M}$, 10 min) shifts the PPD to PPF in ethanol-treated mice ($n = 5$; $p < 0.05$) without affecting the PPF in saline-treated mice ($n = 5$). *d*, IPSC2/IPSC1 ratio plotted as a function of time in cells recorded from saline- and ethanol-treated mice ($n = 5$ per group; $*p < 0.05$) and normalized against the mean of the first 10 min for each cell.

GABA_A IPSCs (Johnson and North, 1992b; Hausser and Yung, 1994). Therefore, to test the possibility that increased probability of GABA release might raise GABA levels and thus activate presynaptic GABA_B receptors, we measured the PPR in the presence of the GABA_B receptor antagonist CGP35348 ($100 \mu\text{M}$, 5 min). Figure 4, *a* and *b*, shows that CGP35348 significantly shifted the PPD to PPF in ethanol-treated animals (IPSC2/IPSC1 = 0.7 ± 0.04 – 1.2 ± 0.08 ; $n = 10$; $p < 0.05$) by increasing the amplitude of the second evoked GABA_A IPSC (IPSC2, $140 \pm 9\%$; data not shown) without affecting either GABA_A IPSC in the saline-treated animals (IPSC2/IPSC1 = 1.5 ± 0.1 – 1.4 ± 0.2 ; $n = 5$; $p > 0.05$). In addition, both the frequency and the amplitude of mIPSCs were unaffected by CGP35348 ($100 \mu\text{M}$, 5 min) in both groups of mice (frequency: ethanol, 2.9 ± 0.2 – 2.6 ± 0.3 Hz; $n = 7$; saline, 1.1 ± 0.1 – 1.1 ± 0.1 Hz; $n = 7$; amplitude: ethanol, 33.6 ± 1.8 – 33 ± 2.9 pA; $n = 5$; saline, 33.2 ± 2.1 – 26.9 ± 2.6 pA; $n = 7$; Fig. 4*c,d*). Thus, the PPD observed in the ethanol-treated mice could result from an increased probability of GABA release, which might in turn lead to activation of presynaptic GABA_B receptors and decrease the IPSC2.

An alternative interpretation of the present results is that the sensitivity of presynaptic GABA_B receptors might be enhanced in the ethanol-treated animals. Therefore, we tested differences in sensitivity of presynaptic GABA_B receptors by comparing the inhibition caused by the GABA_B receptor agonist baclofen in

slices from both saline- and ethanol-treated animals. Figure 5*a* shows that the concentration–response curves to baclofen were similar in the saline- and ethanol-treated mice (baclofen $0.1 \mu\text{M}$: ethanol, $22.3 \pm 8.9\%$; $n = 5$; saline, $25.4 \pm 3.1\%$; $n = 5$; baclofen $1 \mu\text{M}$: ethanol, $42.8 \pm 12.3\%$; $n = 5$; saline, $44.5 \pm 2.3\%$; $n = 5$; baclofen $10 \mu\text{M}$: ethanol, $70.6 \pm 11.8\%$; $n = 5$; saline, $72.7 \pm 7.2\%$; $n = 5$). Because the amplitude of the IPSC1 was decreased in both groups of animals to the same extent at all doses tested (Fig. 5*a,b*), we concluded that the increase in the IPSC2 observed in the presence of CGP35348 in ethanol-treated animals did not result from altered sensitivity of GABA_B receptors to endogenous GABA. Additionally, a high dose of baclofen ($10 \mu\text{M}$; Fig. 5*c,d*) reverted the PPD to PPF in ethanol-treated mice (IPSC2/IPSC1 = 0.7 ± 0.02 – 1.2 ± 0.1 ; $n = 5$; $p < 0.05$), but it produced a nonsignificant increase in PPF in saline-treated animals (IPSC2/IPSC1 = 1.2 ± 0.1 – 1.3 ± 0.1 ; $n = 5$). These results further support the hypothesis that GABA levels are increased after ethanol exposure, leading to spillover onto presynaptic GABA_B receptors, whose activation leads to inhibition of release (Hausser and Yung, 1994). Because changes in the paired-pulse ratio generally reflect changes in the probability of release, we expected baclofen to increase PPF in the saline group. However, we did detect a nonsignificant increase in the PPF during the application of baclofen at this concentration (IPSC2/IPSC1 = 1.2 ± 0.09 – 1.3 ± 0.02 ; $n = 5$; $p > 0.05$) and at $1 \mu\text{M}$ (IPSC2/IPSC1 = 1.1 ± 0.07 – 1.2 ± 0.08 ; $n = 5$; $p > 0.05$) in the saline-treated animals. This indicates that the PPR measure is not sensitive enough in this range to detect a decrease in GABA release, possibly as a result of a ceiling effect.

An increase in probability of GABA release has been described previously during acute withdrawal from chronic morphine in several brain regions, including the VTA (Bonci and Williams, 1997). In particular, this phenomenon has been characterized as being cAMP-dependent in the VTA (Bonci and Williams, 1997), the periaqueductal gray (Ingram et al., 1998), the NAcc (Chieng and Williams, 1998), and the dorsal raphe nucleus (Jolas et al., 2000). Ethanol and other drugs of abuse are known to modulate the cAMP–protein kinase A (PKA) cascade within the mesolimbic system (Hoffman and Tabakoff, 1990; Self et al., 1998; Spanagel and Weiss, 1999). Therefore, to examine the possibility that the cAMP-dependent pathway was modified in the ethanol-treated animals, we directly activated adenylyl cyclase (AC) by bath applying forskolin. Forskolin ($10 \mu\text{M}$, 10 min) augmented the IPSC1 in saline-treated animals (Fig. 6*a*; $111.6 \pm 17.4\%$; $n = 5$; $p < 0.05$) but had no effect on the amplitude of the IPSC1 in ethanol-treated mice ($23.1 \pm 13.3\%$; $n = 5$). Thus, application of forskolin decreased the paired-pulse ratio toward depression in slices from saline-treated animals (Fig. 6*b*; IPSC2/IPSC1 = 1.4 ± 0.1 – 0.9 ± 0.1 ; $n = 5$; $p < 0.05$) but was without effect in slices from ethanol-treated animals (IPSC2/IPSC1 = 0.8 ± 0.05 – 0.7 ± 0.1 ; $n = 5$). This supports the idea that activation of AC increased the probability of GABA release. In addition, the frequency of spontaneous mIPSCs was also significantly increased by forskolin in saline-treated mice (0.7 ± 0.1 – 1.7 ± 0.3 Hz; $n = 7$; $p < 0.05$; Fig. 6*c*) but not in the ethanol-treated animals (2.6 ± 0.7 – 2.9 ± 1.0 Hz; $n = 8$; Fig. 6*c*). There was no significant difference in the amplitude of the mIPSCs in the absence or presence of forskolin in slices from either group of animals (saline, 29.4 ± 2.5 – 31 ± 2.9 pA; $n = 7$; ethanol, 34 ± 4.2 – 26.5 ± 1.7 pA; $n = 8$; Fig. 6*d*). To rule out the possibility of nonspecific effects of forskolin, we tested 1,9-dideoxyforskolin, an inactive analog of forskolin (Seamon and Daly, 1985). Superfusion of 1,9-dideoxyforskolin

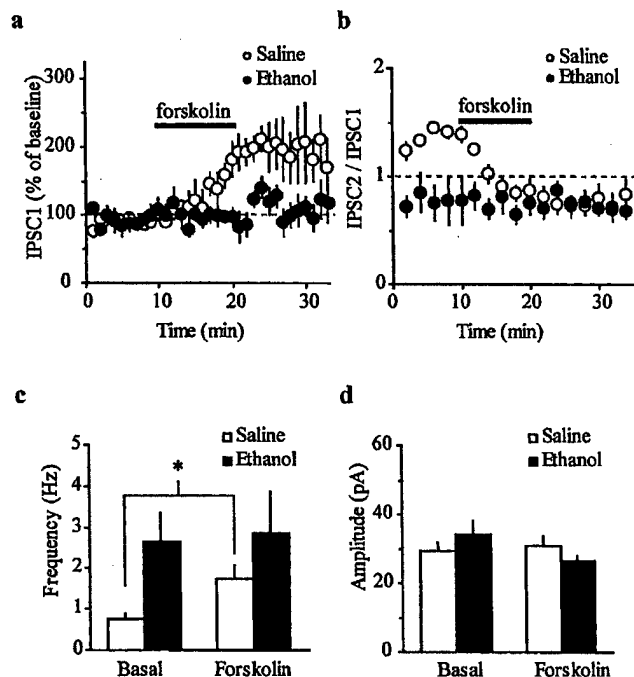


Figure 6. Effect of forskolin on evoked and spontaneous IPSCs. *a*, Forskolin (10 μ M, 10 min) increases the amplitude of evoked IPSC1 in saline-treated mice ($n = 5$; * p < 0.05) but not ethanol-treated mice ($n = 5$). *b*, Forskolin (10 μ M, 10 min) shifts the PPD to PPF in saline-treated mice ($n = 5$; * p < 0.05) without affecting the PPD in ethanol-treated mice ($n = 5$). The IPSC2/IPSC1 ratio is plotted as function of time in cells recorded from saline- and ethanol-treated mice and normalized against the mean of the first 10 min for each cell. *c*, Forskolin (10 μ M, 10 min) induces a significant increase in the frequency of mIPSCs in saline-treated mice ($n = 7$; * p < 0.05) but not in ethanol-treated mice ($n = 9$). *d*, No changes in amplitude were found in either group.

(10 μ M, 10 min) had no effect on IPSCs in either group of animals (7.7 ± 3.3 and $1.1 \pm 2.6\%$ in ethanol- and saline-treated mice, respectively; $n = 4$ for each group; data not shown). Taken together, these results suggest that a saturation of AC might occur after a single *in vivo* exposure to ethanol.

Because changes of intracellular cAMP levels subsequently alter PKA activity, we tested the effect of *N*-[2-(*p*-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H89), which inhibits PKA in a competitive manner against ATP (Chijiwa et al., 1990). Superfusion of H89 (10 μ M, 20 min) significantly reduced the amplitude of IPSC1 in ethanol-treated mice ($38.6 \pm 5.4\%$; $n = 5$; * p < 0.05; Fig. 7*a*) but had no effect in slices from control animals ($3.4 \pm 8.1\%$; $n = 5$). The subsequent application of forskolin (10 μ M, 10 min) in the presence of H89, used to test the activity of H89 in control animals, did not change the amplitude of the IPSC1 in either group (Fig. 7*a*). Furthermore, H89, by reducing the size of IPSC1, shifted the PPD to PPF in slices from ethanol-treated animals (IPSC2/IPSC1 = 0.8 ± 0.02 – 1.1 ± 0.01 ; $n = 5$; * p < 0.05; Fig. 7*b*). Consistent with these results, H89 reduced the frequency of spontaneous mIPSC in the ethanol-treated animals (2.6 ± 0.1 – 1.6 ± 0.1 Hz; $n = 6$; * p < 0.05; Fig. 7*c*) but not in the saline-treated animals (0.9 ± 0.1 – 0.8 ± 0.1 Hz; $n = 5$); in the presence of H89, the amplitude of spontaneous mIPSCs was not changed in either saline-treated mice (31.7 ± 5.2 – 31.3 ± 2.6 pA; $n = 5$) or ethanol-treated mice (28.7 ± 3.1 – 24.8 ± 2.9 pA; $n = 6$). In conclusion, these experiments indicate that PKA activity is significantly enhanced by a single exposure to ethanol,

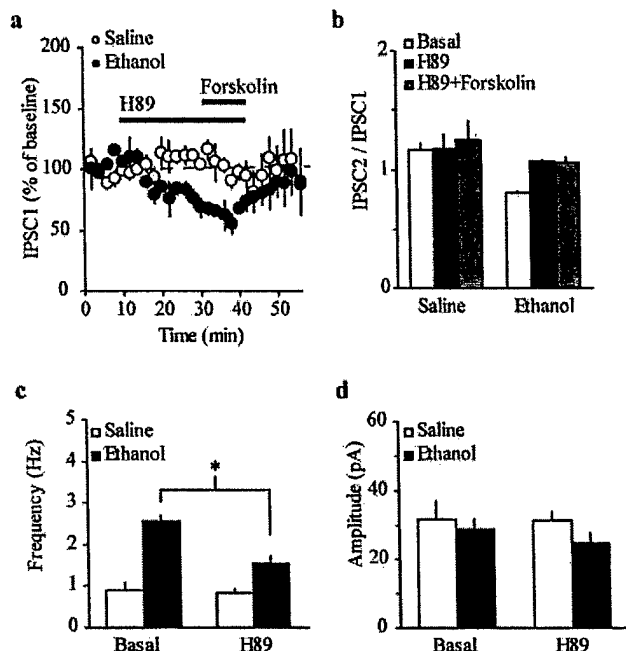


Figure 7. Effect of H89 on evoked and spontaneous IPSCs. *a*, H89 (10 μ M, 20 min) decreases the amplitude of evoked IPSCs in ethanol-treated mice ($n = 5$; * p < 0.05), but not saline-treated mice ($n = 5$). *b*, H89 (10 μ M, 20 min) shifts the PPD to PPF in ethanol-treated mice ($n = 5$; * p < 0.05) without affecting the PPF in saline-treated mice. *c*, H89 (10 μ M, 20 min) induces a significant decrease in the frequency of mIPSCs in ethanol-treated mice ($n = 6$; * p < 0.05) but not saline-treated mice ($n = 5$). *d*, No changes in amplitude were found in either group.

and that such a phenomenon increases the probability of GABA release in the VTA.

DISCUSSION

In the present study, we observe that a single *in vivo* exposure to ethanol produces a long-lasting increase in the probability of GABA release in the VTA, and that such an increase is dependent on the activation of the cAMP–PKA signaling cascade. We hypothesize that this type of plasticity may play an important role in determining the increased alcohol consumption observed after a single exposure to ethanol (Spanagel and Weiss, 1999; Camarini et al., 2000). Our data, together with the fact that we and others have observed increased ethanol consumption when mice were pre-exposed to ethanol, indicate that increased probability of GABA release and increased ethanol self-administration, both produced by the single ethanol injection, might be strictly associated. Indeed, activation of GABA_A receptors plays a role in ethanol self-administration, because GABA_A agonists facilitate acquisition of voluntary ethanol drinking in rats (Smith et al., 1992; Nowak et al., 1998). Accordingly, a role of GABA_A receptors within the VTA in mediating ethanol intake has been suggested (Samson et al., 1987; Boyle et al., 1993; Rassnick et al., 1993b). Indeed, systemic administration of GABA_A receptor antagonists reduces intake (Boyle et al., 1993) and operant responding for ethanol in rats (Samson et al., 1987; Rassnick et al., 1993b). Consistent with these and previous findings, intra-VTA infusions of GABA_A receptor antagonists decreased ethanol consumption in rats of the alcohol-preferring P line (Nowak et al., 1998). To further support the hypothesis that increased GABAergic activity produced by a single *in vivo* exposure to ethanol plays

a role in ethanol-related behaviors, it has recently been shown that there is a direct relationship between pretreatment with ethanol and enhanced self-administration of ethanol in mice (Camarini et al., 2000). Specifically, C57BL/6J mice pre-exposed to ethanol exhibited a significant increase of ethanol intake, and DBA/2J mice, which normally avoid oral ingestion of ethanol, did start to self-administer ethanol in a two-bottle choice test.

In our first set of experiments, we show that a single injection of ethanol shifted the paired-pulse modulation of GABA_A IPSCs from PPF to PPD. The paired pulse stimulation is typically used as an electrophysiological protocol to test for changes in probability of transmitter release (Zucker, 1989; Stuart and Redman, 1991; Manabe et al., 1993; Mennerick and Zorumski, 1995; Debanne et al., 1996). Although this phenomenon is not always use-dependent (Brody and Yue, 2000; Kraushaar and Jonas, 2000; Waldeck et al., 2000), a variety of manipulations that increase transmitter release, including exposure to drugs of abuse, have been found to shift the paired-pulse ratio from facilitation toward depression in the hippocampus (Mennerick and Zorumski, 1995; Salin et al., 1996) and the VTA (Bonci and Williams, 1997). Furthermore, the persistence of PPD 1 week after the ethanol injection suggests that increased GABA_A-mediated inhibition may be considered a measure of changes occurring at these synapses, eventually contributing to the expression of ethanol-seeking behavior.

However, an increase in the probability of GABA release might simply be one of many factors determining the shift from PPF to PPD in the ethanol-treated animals. Although a desensitization of postsynaptic GABA_A receptors could account for PPD, we tend to rule out that possibility, because the ethanol-treated animals show an increase in mIPSCs frequency but not in amplitude when compared with the saline-treated animals. Furthermore, bath application of GABA, in the presence of a GABA_B receptor antagonist, produces similar responses in saline- and ethanol-treated animals.

An alternative explanation for the observed PPD in the ethanol-treated animals, is that activation of presynaptic GABA_B receptors might occur as a consequence of increased GABA levels produced by the first evoked stimulus, thus reducing the amplitude of IPSC2. Indeed, it has been shown that activation of GABA_B receptors inhibits GABA_A IPSCs in the midbrain via a presynaptic mechanism and therefore are considered to serve also as autoreceptors (Hausser and Yung, 1994). Our results showing that the GABA_B antagonist CGP35348 shifted PPD to PPF in animals injected with ethanol, but not in the saline controls, indicate that presynaptic GABA_B receptors might play a minor role when probability of GABA release is relatively low, as in the saline-injected animals. However, they might act as a negative feedback mechanism to regulate GABAergic transmission within the VTA when probability of GABA release is increased, such as after a single *in vivo* exposure to ethanol. Thus, our data indicate that increased GABA levels, by changing the spatial range of synaptically released GABA, allow the activation of presynaptic GABA_B receptors located on the GABAergic interneurons, which in turn would prevent excessive GABA_A-mediated synaptic transmission (McCarren and Alger, 1985; Deisz and Prince, 1989; Davies et al., 1990; Isaacson et al., 1993).

Our results also suggest that the ethanol-induced increase in the probability of GABA release was a result of saturation of the AC cascade within GABAergic terminals. Forskolin, which enhanced the amplitude of evoked IPSCs and the frequency of mIPSCs in saline-treated mice, had no effect in the ethanol-

treated mice. In addition, the PKA inhibitor H89 reduced the amplitude of evoked IPSCs and the frequency of mIPSCs only in ethanol-treated animals, whereas it had no effect in saline-treated animals. These findings suggest that a single *in vivo* exposure to ethanol results in persistent enhancement of PKA-dependent processes in GABAergic terminals in the VTA. It is possible that because of high cAMP levels after the exposure to ethanol, the catalytic subunits of the PKA complex become unbound and freely diffuse within the terminals. In fact, in slices from ethanol-treated animals, H89 revealed an increased basal activity of PKA, and the activation of AC by forskolin was blunted. Consistent with our findings, reduced signaling through the cAMP–PKA system, whether because of decreased expression of the α subunit of the stimulatory G-protein ($G_{s\alpha}$) or inhibition of PKA, changed C57BL/6J mice, considered to be an ethanol-preferring line of mice, into ethanol nonpreferring mice (Wand et al., 2001). In addition, alcohol-preferring rats show increased AC activity and expression of $G_{s\alpha}$ in mesolimbic regions when compared with alcohol-nonpreferring rats (Froehlich and Wand, 1997). More generally, our results are in agreement with previous studies reporting that genetic manipulations of the cAMP–PKA pathway modulate ethanol intake and sensitivity to its sedative effects (Thiele et al., 2000; Wand et al., 2001). Although the relationships between the sedative and rewarding effects of ethanol are complex, it is also important to mention that the cAMP–PKA system has been implicated in neural plasticity associated with drug tolerance and dependence (Self and Nestler, 1995; Moore et al., 1998; Andretic et al., 1999; Yoshimura and Tabakoff, 1999). Finally, it has been shown that chronic exposure to many drugs of abuse, including ethanol, also leads to increased activity of cAMP-dependent processes (Terwilliger et al., 1991; Dohrman et al., 1996; Bonci and Williams, 1997).

In conclusion, our results provide evidence that a single *in vivo* exposure to ethanol produces a long-lasting potentiation of GABAergic synapses in the VTA. This cAMP–PKA-dependent plasticity occurring at these synapses might represent an important cellular signaling event underlying increased ethanol consumption. Whether these changes are the result of a compensation for acute effects of ethanol or manifestation of a long-lasting effect of acute ethanol remains to be elucidated. Although acute effects of ethanol on the GABAergic systems in the CNS are still a matter of debate, we tend to support the latter possibility, because ethanol has been found to enhance GABAergic transmission in several brain regions (Celentano et al., 1988; Deitrich et al., 1989; Aguayo and Pancetti, 1994; Mehta and Ticku, 1994; Wan et al., 1996; Nie et al., 2000), including the VTA (M. Melis and A. Bonci, unpublished observations). In conclusion, we observed that a single *in vivo* ethanol exposure induces a long-lasting potentiation of GABA synaptic transmission within the VTA via a cAMP–PKA mechanism, and that it appears to be directly related to increased ethanol consumption and may therefore be involved in the development of alcoholism.

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